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REPORT NO. 599

THE CHEMICAL MODIFICATION OF NECROGENIC AND PROTEOLYTIC ACTIVITIES OF CROTALIDAE VENOM AND THE USE OF EDTA TO PRODUCE A VENOM TOXOID

Charles R. Goucher, Ph. D.
Captain Herschel H. Flowers, VC

Department of Biochemistry
Division of Medicine
US ARMY MEDICAL RESEARCH LABORATORY
Fort Knox, Kentucky

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ABSTRACT

THE CHEMICAL MODIFICATION OF NECROGENIC AND PROTEOLYTIC ACTIVITIES OF CROTALIDAE VENOM AND THE USE OF EDTA TO PRODUCE A VENOM TOXOID

OBJECTIVE

To investigate the proteolytic activity, necrolytic activity, and antigenicity of untreated venom and venom which had reacted with ethylenediaminetetraacetic acid (EDTA).

RESULTS AND CONCLUSIONS

Agkistrodon piscivorus venom treated with EDTA lost ability to digest casein. Neither sodium phosphate nor sodium bicarbonate restored this ability. The hemorrhagic and necrotizing activity of this venom was essentially absent following EDTA treatment. EDTA treated venom which did not produce necrosis and hemorrhage stimulated the production of antibodies which neutralized the local and systemic toxic activity of untreated venom.

RECOMMENDATIONS

The usefulness of EDTA in the treatment of snakebite should be investigated and attempts should be made to isolate and characterize the necrogenic and proteolytic components of snake venom.

APPROVED: Fred D. Maurer
FRED D. MAURER
Colonel, VC
Director, Division of Medicine

APPROVED: Sven A. Bach
SVEN A. BACH
Colonel, MC
Director

THE CHEMICAL MODIFICATION OF NECROGENIC AND PROTEOLYTIC ACTIVITIES OF CROTALIDAE VENOM AND THE USE OF EDTA* TO PRODUCE A VENOM TOXOID

I. INTRODUCTION

The important observation of Deutsch and Diniz that EDTA inhibited venom proteinases has suggested the existence of a unique class of proteolytic enzymes, and apparently an essential role of metals in the activities of venom toxins (1, 2).

This communication demonstrates the suppression of the necrotizing properties of Crotalid venoms by EDTA in the rabbit. The effects of various parameters on the course of venom inactivation by EDTA are reported; furthermore, venom treated with EDTA was shown to induce antibody formation against the necrotizing and toxic effects of native venom.

II. MATERIALS AND METHODS

Proteolytic activity was determined by the casein digestion method of Kunitz (3). Digestion was carried out for 20 minutes at 35°C at a casein concentration of 0.5% in 0.05 molar Tris-HCl** buffer at pH 8, if not indicated otherwise. Identical blanks were used in which the mixing of all reactants was quickly followed by the addition of trichloroacetic acid. Proteolytic activity was terminated by the addition of 3 ml of 10% TCA to 2 ml of reaction mixture. The suspension thus obtained was filtered after 30 minutes and the optical density of the resulting filtrate was measured at 2800 Å against the appropriate blank.

Immunodiffusion analyses were carried out by placing 15 ml of sterile Difco agar in plastic petri dishes of 10 cm diameter. The agar was solidified in the cold and a center well 1 cm in diameter was cut. Two to four peripheral wells, 0.8 cm in diameter were located 4-5 mm from the center well. Solutions containing 10 mg of venom per ml were placed in the center well, and normal saline solutions containing 100 mg per ml of rabbit immunoglobulins were placed in the peripheral wells. The plates were incubated for 3 to 5 days at 22°C and then photographed.

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*EDTA, disodium salt of ethylenediaminetetraacetic acid.

**Tris-, tris (hydroxymethyl)aminomethane.

The ability of rabbit antibodies to neutralize the hemorrhagic activity of venom was determined by a method which was essentially that of Kondo (4). A saline solution containing 2 mg per ml of venom was added in equal parts to physiological saline containing 100 mg per ml of rabbit immunoglobulin. As a control, 1 mg per ml of untreated venom in saline was prepared. Solutions with and without antibody were incubated at 37°C for 1 hour before injection and 0.1 ml of the venom-globulin mixture and 0.1 ml of the control were injected intradermally into a depilated rabbit. The animals were sacrificed 24 hours after injection and the skin was removed and the undersurface was photographed. Neutralization was indicated by comparison of the intradermal hemorrhage at the control and at the area in which venom was treated with antibody.

The titer of gamma globulin for venom toxins in the two groups of animals was determined by the method of Gingrich and Hohsnadel (5). Venom was dissolved in normal saline in a concentration of 20 LD_{50} per ml for 18 to 20 gm female Swiss mice. Lyopholized rabbit globulin was dissolved in saline to a concentration of 100 mg per ml and serial dilutions of this solution were prepared. A given quantity of venom and serial dilutions of gamma globulin were combined and incubated for 30 minutes at 22°C; 0.5 ml of these solutions were injected intravenously into mice. Death of the animal within 24 hours after injection was attributed to venom toxicity.

To compare the venom antigens of EDTA treated venom with those of untreated venom, two groups of eight rabbits were immunized. A series of eight injections, increasing in amounts, was given each animal as described by Russell (6). Venom was dissolved in normal saline and added to an equal quantity of 4% sodium alginate. The injection site was the subcutaneous region of the shoulder. Ten days following the last injection the animals were exsanguinated and their serum collected. Globulins were collected by precipitation with 35% ammonium sulfate. The precipitates were dialyzed and lyopholized. During the course of immunization, the group of animals injected with untreated venom suffered 20% mortality and the surviving animals receiving untreated venom suffered severe local reactions and sloughing of tissue. Neither deaths nor sloughing occurred in the group of animals immunized with EDTA treated venom. There were tissue reactions in all animals and septic abscesses developed in two animals receiving EDTA treated venom. These were controlled by surgical draining and antibiotic therapy.

III. RESULTS

Casein Digestion. The digestion of casein by various concentrations of venoms, during a 20-minute interval at 35°C, is shown in Figure 1. This curve served as a standard in assessing the effects of ions and chelating agents on proteolytic activity in reaction mixtures differing with respect to venom activity, ion content, and concentration.

The Temperature, Time, and Concentration Dependence of Proteinase Inactivation by EDTA. The time required, at 35°C, for different concentrations of EDTA to alter the level of activity of the venom enzymes which digest casein is shown in Figure 2. The plateaus observed between 0.66 mM EDTA and 1.65 mM EDTA appear to indicate the presence of at least two venom enzymes which digest casein and which differ in their sensitivity to EDTA inactivation.

A similar pattern of inactivation was observed when the digestion time was doubled. Casein was digested by both enzymes and the rate of digestion during the second 20 minute interval did not differ greatly from that observed during the first 20 minute interval.

Venom toxoids were produced by reacting venom with 10^{-2} molar EDTA at 35°C for 2 hours at pH 7.5. The proteolytic activity of venom on casein following this treatment is shown in Table 1. In order to determine the weakened proteolytic activity of venom preparations used as antigens, casein digestion was continued up to 30 minutes. These data show that casein was digested following the treatment indicated, and show again the existence of enzymes which digest casein and which are comparatively resistant to EDTA inactivation.

EDTA inactivation of venom proteinases was temperature dependent. The extent to which temperature influenced the inactivation of these enzymes by EDTA is shown in Figure 3.

The Digestion of Casein by Venom Proteinases in the Presence of Combinations of NaCl, $NaHCO_3$, and EDTA. The data in Table 2 show the extent to which casein digestion was altered by the presence of sodium bicarbonate, and by sodium chloride in solutions with and without EDTA. In the preparation of sodium bicarbonate solutions, the hydrogen ion concentration was not permitted to drop below pH 8 (2). In testing for stimulation of proteolytic activity or for the alleviation of EDTA inhibition, bicarbonate solutions were added to

solutions of casein or venom which were buffered at pH 8. The various components tested were reacted with venom at 35°C for 15 minutes, and the final concentration of EDTA in these solutions was 3.3 mM.

Differences in casein digestion in the presence and absence of bicarbonate are of a magnitude which fall within the limits of experimental error. Neither $NaHCO_3$ nor NaCl produced a significant alteration in EDTA inhibition.

The effect of phosphate on the EDTA inhibition of venom proteinases was reinvestigated. The data in Figure 4 show that under the conditions of this experiment the addition of a second possible ligating substance to solutions of venom and EDTA increases rather than diminishes the proteinase activity of such solutions.

The Effect of EDTA on the Ability of Venom to Produce Intradermal Necrosis. A typical hemorrhagic lesion produced by the intradermal injection of venom is marked as control in Figure 5. This hemorrhagic area was produced by 500 μg of venom injected in 0.1 ml of saline. This quantity of venom gave an optical density of only 0.39 at 2800 Å in the standard proteolytic assay.

The hemorrhagic areas shown in the top row of Figure 5 were each produced by the same quantity and volume of venom used for the "control" but the venom used for injection in the areas marked with time intervals were incubated with EDTA. The time intervals denote the length of time venom reacted with EDTA at 35°C before injection.

A Comparison of Immunochemical Reactions of Untreated Venom and the Immunoglobulins of Rabbits Inoculated either with Untreated Venom or with EDTA Treated Venom. The gross similarity among immunoglobulins from rabbits immunized with EDTA treated venom and untreated venom was revealed in the immunodiffusion pattern shown in Figures 6a and 6b. The prominent confluence of numerous precipitation bands emphasized the similarity in antigenic structure of the non-necrotizing EDTA treated venom and the highly necrotizing untreated venom.

This similarity in antigenic structure was emphasized again by the observation (Fig. 7) that antiserum from a rabbit immunized with the non-necrotizing EDTA treated venom neutralized the necrogenic activity of untreated venom. Using the intradermal skin test, the

antiserum produced in response to the EDTA treated venom was indistinguishable from the antiserum produced in response to the untreated necrotizing venom.

The ability of antisera to neutralize the lethal toxicity of venom injected intravenously was investigated in the mouse. A group of rabbits were inoculated with untreated venom and another group was prepared by 35% $(NH_4)_2SO_4$ precipitation from the serum of each group and it was dialyzed, lyopholized, and finally mixed in solution with untreated venom. The LD_{50} of these solutions are compared in Table 3. It was observed that the immuno-response to EDTA treated and untreated venom with respect to neutralization of lethal toxicity was essentially the same. The responses observed with both types of venom distinguished the sera of the inoculated group from the sera of rabbits which were not inoculated with venom.

IV. DISCUSSION

The proteolytic activity of Crotalid venom is inhibited by chelating agents (1), but this activity is not significantly diminished by dialysis. These observations suggest that the enzymes of venom are metalloproteins rather than metal protein complexes (7).

Inhibition of metalloprotein enzymes by chelating agents may occur in consequence of the displacement of an essential metal from the enzyme by the chelating agent and in consequence of the occupancy of an essential coordinate position on the protein bound metal by the chelating agent. In both mechanisms of inactivation the ligand, chelating agent, functions as an electron donor and the metal as an electron acceptor. From these considerations it would appear unlikely that inhibition by a given ligand would be reversed by the addition of a second ligand.

Phosphate and sodium bicarbonate, like EDTA, may act as ligands and in this capacity react with or remove enzyme bound metal. Yet, Philpot and Deutsch (2) and Philpot (8) state that phosphate and sodium bicarbonate alleviate (1) inhibition of venom proteinases by EDTA.

Because of the difficulty in visualizing a mechanism which would account for such an antagonism of EDTA inhibition by other ligands, the question of the effect of EDTA on venom proteinases was reinvestigated.

The inhibition of enzyme activity by a chelating agent is presumptive evidence that a metal is essential to that activity. It may be imagined that a given metalloenzyme would evince a characteristic inactivation rate at various concentrations of chelating agents. On this basis, the data in Figure 2 show that at least two enzymes or groups of enzymes are active in the digestion of casein by venom. These enzymes are distinguished by a difference in their sensitivity to EDTA inactivation, and this difference appears as the sharp break in the curves of Figure 2, and in the broad plateaus apparent at EDTA concentration above 0.66 mM.

In this study, contrary to the reports of others (1), EDTA did not completely inhibit the proteinases of the cottonmouth moccasin. In an attempt to produce a venom toxoid which caused little or no dermal necrosis, venom was reacted with relatively high concentrations of EDTA for several hours at 37°C, but this venom continued to show proteolytic activity on casein (Table 1) although it was minimally necrogenic.

The strong temperature dependence of EDTA inactivation (Fig. 3) may suggest that loss of activity, if the chelating agent reacted rapidly with the metal of the enzyme, results from a denaturation represented by a conformational change in the enzyme. The use of radioactive labeled EDTA in a study of its possible fixation to enzyme protein may be of value in elucidating the mechanism of this inactivation, as well as elucidating the strength of a possible metal to protein bond.

It has been reported that EDTA inhibition of venom proteinases was reversed by physiologically ubiquitous ions such as phosphate and sodium bicarbonate (1, 8). One conclusion to be drawn from these reports was that while EDTA inactivated some of the toxic components of venom under certain in vitro conditions, it would be unlikely that this chelating agent could inhibit the action of these toxins in vivo. However, as stated above, the notion of the alleviation of EDTA inhibition by other ligands was difficult to rationalize in terms of known chemical mechanisms.

The effect of phosphate and of sodium bicarbonate on venom enzymes which digest casein therefore was reinvestigated. Figure 4 shows the effect of phosphate on the digestion of casein and on the EDTA inhibition of caseinase activity. Here it was apparent that less proteolytic activity was manifested in solutions containing phosphate and EDTA than in solutions containing only EDTA. The presence

of phosphate appeared to potentiate EDTA inhibition rather than reverse such inhibition. The effect of sodium bicarbonate ion and of sodium chloride on EDTA inhibition of venom proteinases was explored. Table 2 shows that EDTA inhibition of these enzymes was reversed neither by sodium bicarbonate alone nor by sodium chloride. In the absence of EDTA casein digestion was unaffected by sodium chloride and by sodium bicarbonate.

In this study, the inability to observe a reversal of EDTA inhibition by phosphate and sodium bicarbonate suggested the possible value of chelating agents in the inactivation of venom in vivo. Preliminary to such an investigation, venom and EDTA were mixed in a test tube and the solution was maintained at 35°C with periodic sampling. These samples of venom with EDTA were injected intradermally at various times after mixing. It is apparent from Figure 6 that venom constituents responsible for intradermal hemorrhage were inactivated by reacting with EDTA and that this inactivation was decidedly time dependent and analogous to proteolytic enzyme inactivation by EDTA.

A major difficulty in the use of native venoms as antigens in the production of potent antivenins is the extensive local hemorrhage which occurs at the site of injection (9). The observation that EDTA treated venom failed to produce extensive hemorrhage suggested the use of this chelating agent for the production of a venom toxoid as well as for the treatment of snakebite (10). It was speculated that the preponderant surface of the metal containing protein molecule might not be altered appreciably by a reaction between the metal and this ligand. If such an extensive alteration did not occur, then antibodies against the non-necrogenic EDTA treated venom might react with native venom.

Untreated venom and venom which had reacted with EDTA were mixed with Na-alginate adjuvant, and rabbits were inoculated with these preparations. Following this treatment serum was obtained from the rabbits with antibodies against EDTA treated venom and against non-treated venom.

The considerable similarity of the antibodies produced by non-necrotizing EDTA treated venom and the necrotizing non-treated venom was revealed by the similarity and confluence of precipitation bands in immunodiffusion analysis (Figs. 6a and 6b) and by the ability of antibodies against EDTA treated venom to neutralize components of native venom which produced local necrosis (Fig. 7) and against those which produced systemic intoxication when injected intravenously (Table 3).

V. SUMMARY

Venom treated with EDTA lost the ability to produce intradermal necrosis.

EDTA treated venom was capable of stimulating the production of antibodies which reacted with and neutralized toxins of untreated venom.

Neither sodium bicarbonate nor phosphate reversed the EDTA inhibition of proteolytic enzymes of Agkistrodon piscivorus venom.

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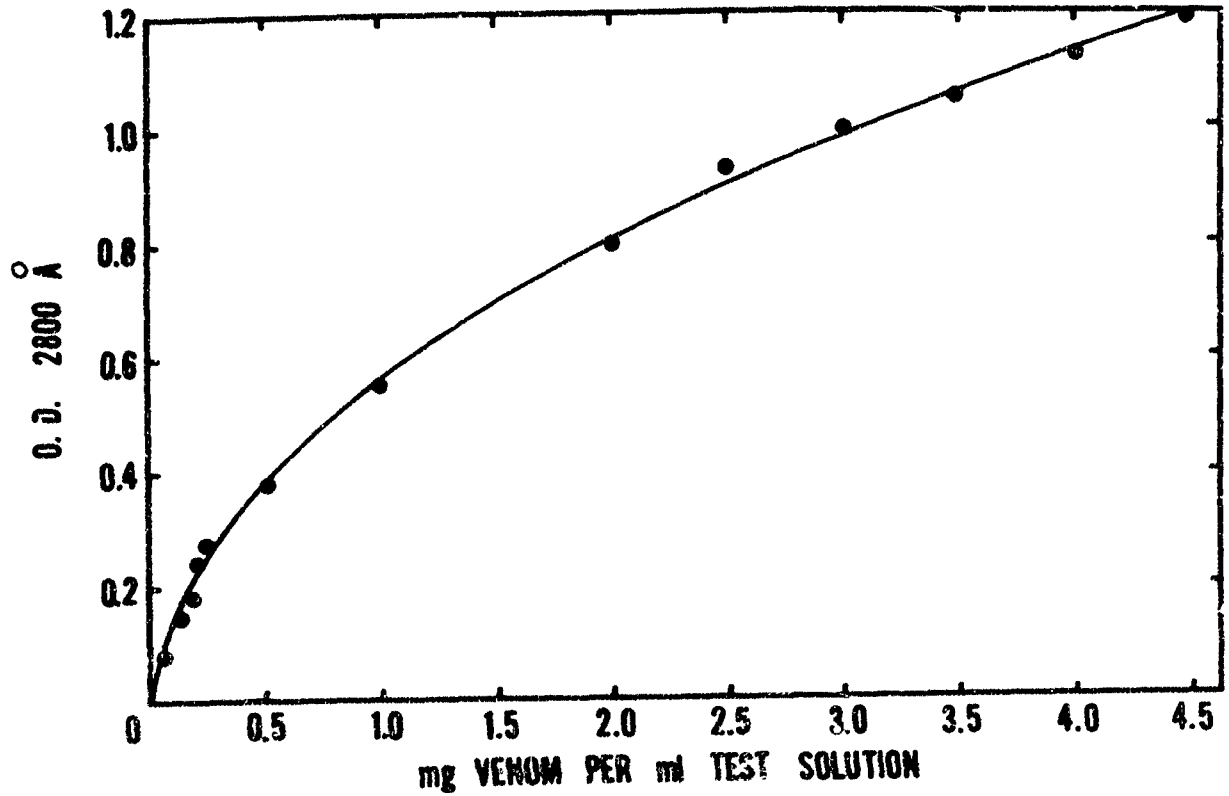

Fig. 1. Digestion of casein by Crotalus venom. Optical density at 2800 Å vs. mg venom protein. Digestion time 20 minutes.

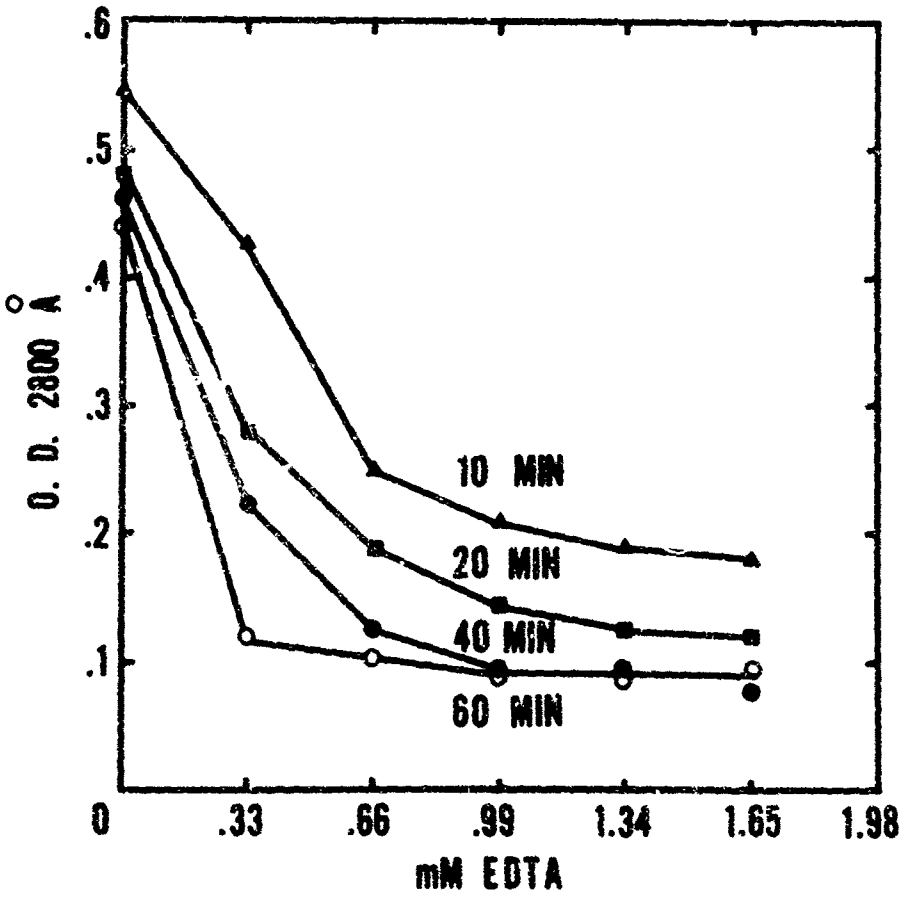

Fig. 2. The inhibition of proteolytic activity of venom as a function of EDTA concentration and as a function of the time of reaction between EDTA and venom before the addition of casein. Reaction mixture: 0.1 ml venom (10 mg/ml H_2O), 0.5 ml 2 molar tris-HCl, and 0 ml to 0.5 ml of 330 mM EDTA or H_2O. After incubation at 35°C with EDTA for the indicated time 1.0 ml of casein in 0.05 M tris-HCl was added and digestion occurred for 20 minutes before the addition of TCA. Venom and casein were together before the addition of casein for the time shown on each curve.

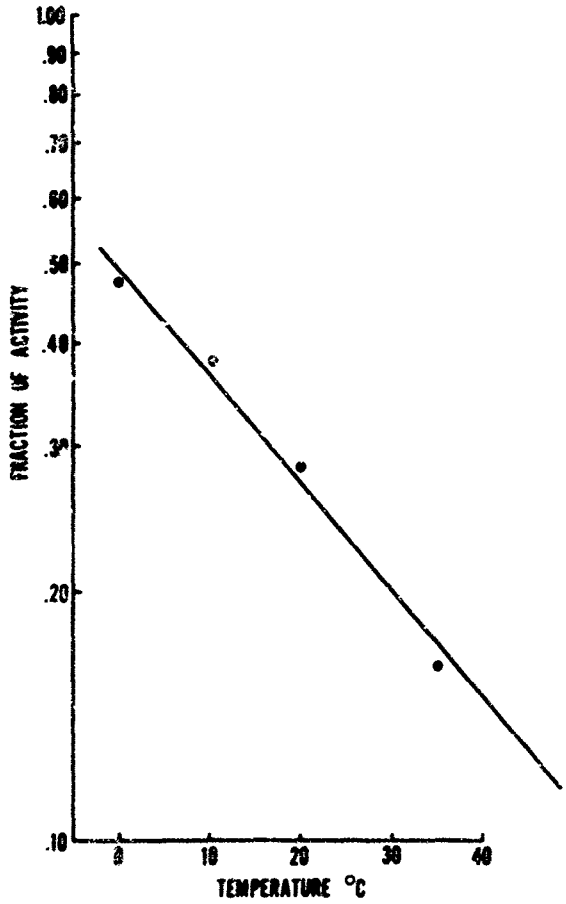

Fig. 3. The effect of temperature on the EDTA inactivation of venom proteinase. EDTA reacted with venom for 1 hour at pH 8 at the temperatures indicated in solutions 2 mM in EDTA containing 7.5 mg of venom per ml. All reactants were dissolved in 0.05 molar tris-HCl buffer and in the control solution buffer was substituted for EDTA. Following incubation aliquots of these solutions were mixed with casein to give reaction mixtures 0.5% in casein, 0.2 mM in EDTA, and containing 0.75 mg venom per ml. The values on the ordinate represent the fraction of proteolytic activity present following incubation with EDTA for 1 hour, and the values on the abscissa show the temperatures at which incubation took place. The points are the average of two determinations and the line is arbitrarily drawn.

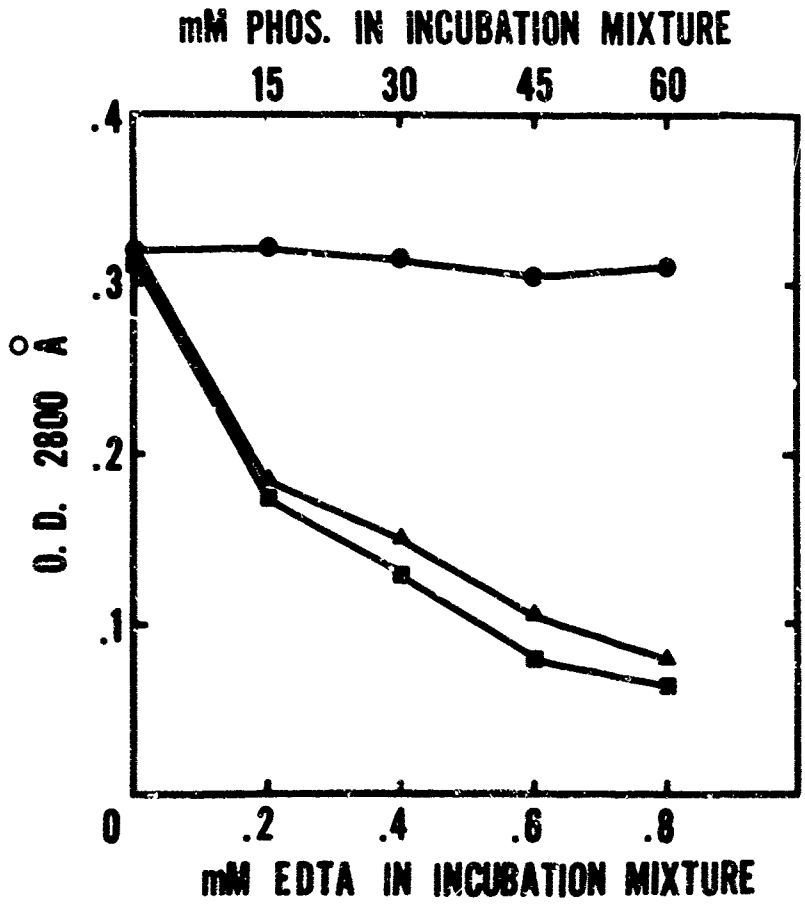

Fig. 4. The effect of phosphate on the EDTA inhibition of venom proteolytic activity. Solutions of phosphate, EDTA, and phosphate with EDTA were prepared. These solutions contained 7.5 mg of venom per ml, and were kept at 35°C for 1 hour. Proteolytic activity was then determined in reaction mixtures containing 0.75 mg venom per ml and 0.5% casein. The values on the ordinate are the optical densities observed in TCA filtrates following 20-minute digestion, and the values on the abscissa show the concentration of EDTA and phosphate in each incubation mixture tested. Phosphate only, ●——●; EDTA only, ▶——▶; and EDTA with phosphate, ■——■.

Fig. 5. The time course of EDTA inactivation of the hemorrhagic activity of snake venom. The lesions in the upper row were produced by 0.5 mg of venom in 0.1 ml of physiological saline, in the lower row one-half of this quantity of venom was used. The venom solutions injected as control contained no EDTA. Venom solutions at 10^{-2} molar EDTA were designated by time intervals which denote the length of the period following the mixing of venom and EDTA at 37°C before injection.

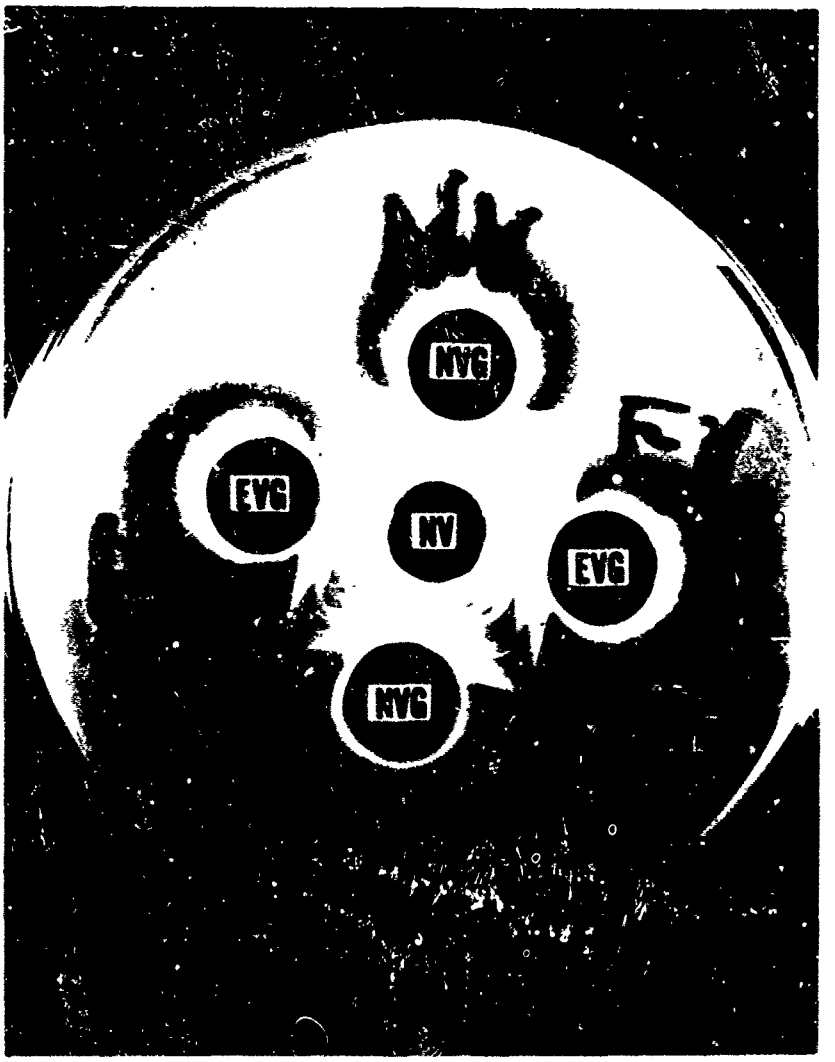

Fig. 6a. An immunodiffusion analysis of antibodies produced by untreated venom and venom treated with EDTA. Solutions of antibodies stimulated by untreated and EDTA treated venom are designated respectively NVG and EVG, the solution of untreated venom is designated NV.

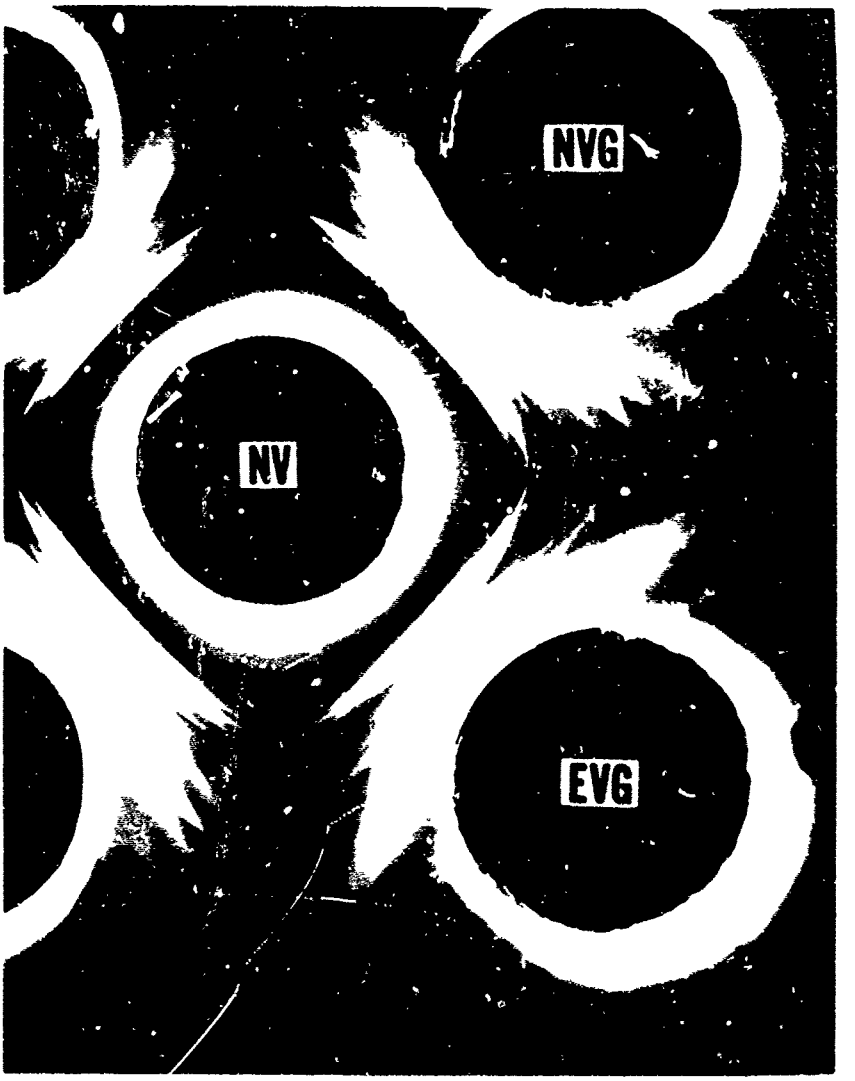

Fig. 6b. An immunodiffusion analysis of antibodies produced by untreated venom and venom treated with EDTA. Solutions of antibodies stimulated by untreated and EDTA treated venom are designated respectively NVG and EVG, the solution of untreated venom is designated NV.

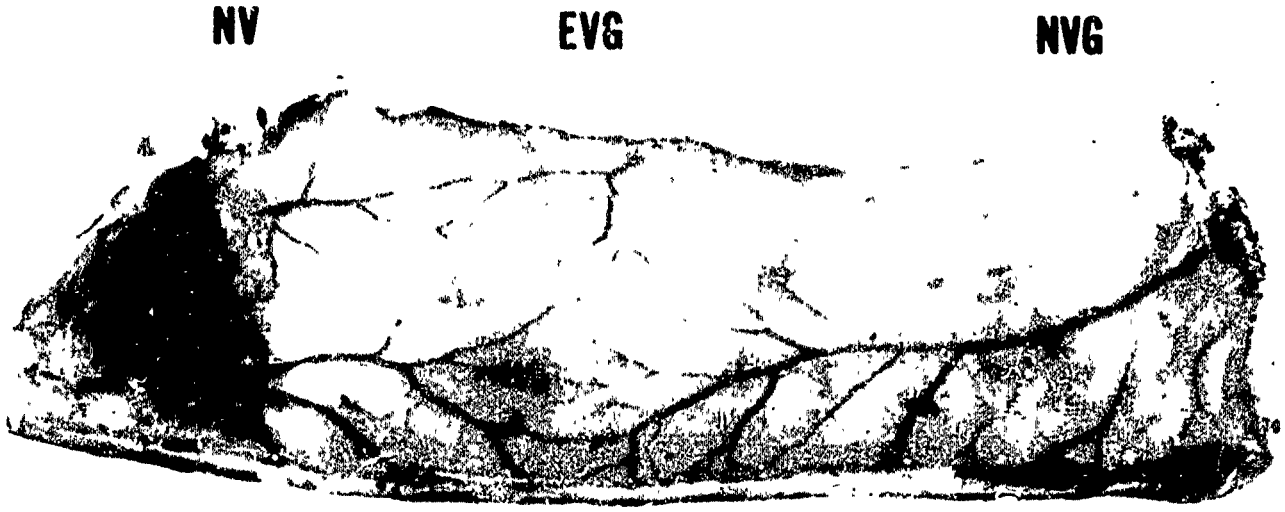

Fig. 7. The neutralization of venom hemorrhagic activity by immunoglobulins produced in response to untreated and EDTA treated venom. Each of the three discrete areas indicated were injected with 1.0 mg of venom, the area below NV contained untreated venom without immunoglobulins, the area below EVG contained untreated venom which had reacted with immunoglobulins produced by EDTA treated venom, and the area below NVG contained untreated venom which had reacted with immunoglobulins produced by untreated venom.

TABLE 1

The Digestion of Casein by Venom Preparations Used as Antigens

Digestion Time Minutes	Mg Venom per ml Reaction Mixture	OD 2800 Å with EDTA	OD 2800 Å no EDTA
20	0.25	.045	.248
"	0.50	.045	.344
40	0.25	.073	.370
"	0.50	.100	.456
60	0.25	.093	.415
"	0.50	.116	.567

A solution of 10^{-2} molar EDTA, pH 8, containing 5.0 mg per ml of venom was incubated 2 hours at 35°C and then proteolytic activity on casein was tested as usual at digestion times indicated above.

TABLE 2

The Effect of Various Combinations of EDTA, $NaHCO_3$, and NaCl on the Digestion of Casein by Moccasin Venom

Venom ml	EDTA ml	$NaHCO_3$ ml	NaCl ml	H_2O ml	2800 Å OD
0.5	0	0	0	0.5	0.28
0.5	0.3	0	0	0.2	0.14
0.5	0.3	0.1	0	0.1	0.16
0.5	0.3	0	0.1	0.1	0.14
0.5	0	0.1	0	0.4	0.26
0.5	0	0	0.1	0.4	0.29

The solutions described above were kept at 35°C for 30 minutes then one part of venom was mixed with one part of 1% casein. 1.0 molar $NaHCO_3$, 1.0 molar NaCl, and 0.1 molar EDTA solutions were used. All reactants were at pH 8 in 0.05 molar tris-HCl. The venom solution contained 1.0 mg of venom per ml.

TABLE 3

Neutralization of the Lethal Toxicity of Venom by Immune Globulin Obtained from Rabbits Inoculated with Untreated and EDTA Treated Venom of Agkistrodon Piscivorus

Globulin Type	Globulin Concentration (mg/ml)	No. Mice Injected	No. Mice Survived
U	50	8	8
U	37	8	0
U	25	8	0
EDTA	50	8	4
EDTA	37	8	0
EDTA	25	8	0

Each solution indicated above contained 10 LD_{50} per ml and mice were injected with 0.5 ml of these solutions. Venom was mixed with globulin and the resulting solutions were kept at 32°C for 30 minutes before injection. One hundred mg of U-globulin neutralized 20 LD_{50} of untreated venom, and 100 mg of EDTA-globulin neutralized 16 LD_{50} of untreated venom. U-globulin was the immune globulin obtained from rabbits inoculated with untreated venom and EDTA-globulin was the immune globulin obtained from rabbits inoculated with EDTA treated venom.

ANNEX [illegible]

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